Master's thesis

Tracing activation efficiency of the bio-filtration bacteria in a Recirculating Aquaculture System using Stable Isotope Analysis

Nathan Foore

University of Jyväskylä

Department of Biological and Environmental Science

International Aquatic Masters Programme

10.10.2016

University of Jyväskylä, Faculty of Science

Department of Biological and Environmental Science
International Aquatic Masters Programme
Foore, Nathan: Tracing activation efficiency of the bio-filtration bacteria in a Recirculating Aquaculture System using Stable Isotope Analysis

Supervisors: Sanni Aalto & Jouni Vielma

October 2016

Keywords: bio-filtration, nitrification, RAS, Stable Isotope Analysis (SIA)

ABSTRACT

One challenge in starting a new recirculation aquaculture system (RAS) is to find the most efficient method to activate the nitrifying bacterial biofilm in the bio-filtration system. It has been suggested that chemical startup with ammonia and nitrite surpasses the "cold start" method where biofilm originates from the fish introduced to the un-activated system. However, there is no information on how the start-up method affects the actual nitrification activity of the bio-filtration system.

The objectives of this study were to evaluate how different activation techniques affect the overall function and the nitrification activity of the bio-filtration units, and the interactions between heterotrophic and nitrifying bacteria in the RAS. The knowledge is poor on how different start up methods affects nitrification activity. All previous studies have focused on ammonium reduction rates and not on the actual nitrification activity process rates. This is the first study to do an in depth analysis of comparing activation techniques used for bio-filtration units in a Recirculating Aquaculture System. Also this is the first study to analyze how the activation technique affected the activity of the bio-filter and the proportion of heterotrophic bacteria vs. nitrifers that were contained in the biofilm by using carbon and nitrogen stable isotope analyses.

To study these questions, I conducted an experiment at the Natural Resources Institute Finland, in Laukaa, Finland. We had eight experimental RAS units, four different start-up methods tested (cold start method with fish, ammonia addition, ammonia + nitrite addition, ammonium + nitrite + glucose addition) with two replicate units per treatment. The experiment was divided to two phases. The activation phase continued for five weeks (Feb 10 - Mar 16, 2016) after which rainbow trout (average size appr. 0.6 kg, biomass 20 kg per tank) where added to the units. Once the fish were added all of the additions in the treatments were stopped. Fish were fed 24 hours per day, at the rate of 1.6% per day. The addition of the fish in all the treatments after activation of the biofilter system was measured for a period of 25 days.

In the activation period, the ammonia levels were highest in the ammonia treatment. After the fish were added, all treatments, besides the treatment with glucose, had an increase in the nitrate concentrations, signaling the nitrification activity. The highest nitrate

concentration was in the cold start treatment followed closely by the ammonia and the ammonia and nitrite treatments. In the glucose treatment, the ammonia levels continued to rise, signaling low nitrification activity. During the activation period, the carbon content of the biofilm in the cold start and glucose treatments was already high, whereas in the ammonia and in the ammonia + nitrite treatments the biofilm carbon content rose rapidly after the addition of rainbow trout, implying the development of the biofilm biomass. The $\delta^{13}C$ of the biofilm in all other treatments than the glucose treatment reflected the isotope value of the faeces and DIC, while in the glucose treatment the δ13C originated from the glucose used by the heterotrophs. The $\delta^{15}N$ of the biofilm correlated positively with nitrate concentration, with highest $\delta^{15}N$ values found from ammonia, ammonia + nitrite and cold start treatments, indicating their highest nitrification potential. In the glucose treatment, the $\delta^{15}N$ of biofilm reflected mainly heterotrophic remineralization of organic matter.

The cold start treatment has been demonstrated to be equally as effective as the chemical startup treatments. Evidence also suggests that the cold start treatment 1) amplifies the nitrification rate of the biofilm in the bio-filtration system, among the tested treatments, and 2) improves maturation time, just as effectively as the chemical startup treatments do. These results may come as a surprise to many who believed in the unassailable superiority of the chemical treatments. As for the glucose-subsidized method, it led to poor nitrification activity, indicating that adding glucose increases the amount of heterotrophic bacteria in the biofilm, which means the nitrifiers are less efficient at nitrification. Combining lab and field work with stable isotope analysis it is possible to come to accurate and detailed findings about the nitrification activity rates of bio-filtration treatments.

JYVÄSKYLÄN YLIOPISTO

Matemaattis-luonnontieteellinen tiedekunta
Bio- ja ympäristötieteiden laitos Akvaattiset tieteet
Foore, Nathan: Biosuodatuksen bakteeritoiminnan käynnistymisen arviointi stabiilien isotooppien avulla kalojen kiertovesikasvatuksessa

Työnohjaajat: : Sanni Aalto & Jouni Vielma

Lokakuu 2016

Keywords: biosuodatus-systeemissä, nitrifikoiviin, stabiilien isotooppien analysointiin (SIA)

TIIVISTELMÄ

Haasteena uuden Vesiviljelyn kiertojärjestelmän (RAS) aloittamisessa on löytää tehokkaimmat menetelmät, joilla aktivoidaan nitrifikoivien bakteerien biofilmi biosuodatus-systeemissä. On ehdotettu, että kemiallinen aloitus ammoniakilla ja nitriitillä ohittaa ”kylmäkäynnistys” – menetelmän, jossa aktivoimattomaan systeemiin lisätty kala tuottaa biofilmin. Toistaiseksi ole kuitenkaan tietoa, kuinka käynnistysmenetelmä vaikuttaa nitrifikaatioprosessiin biosuodatus-systeemissä.

Tämän tutkielman tavoitteena oli arvioida, kuinka erilaiset aktivointitekniikat vaikuttavat yleisesti RAS-systeemin toimintaan, nitrifikoiviin bakteereihin biosuodatusyksiköissä sekä heterotrofisten ja nitrifikoivien bakteerien vuorovaikutukseen. Erilaisten käynnistysmenetelmien vaikutuksista nitrifikaation aktiivisuuteen ei ole toistaiseksi paljoa tietoa. Aikaisemmat tutkimukset ovat keskittyneet ammoniakin vähentymisen asteisiin eikä itse nitrifikaation aktiivisuustasoihin. Tämä tutkielma on ensimmäinen tutkimus, joka tekee syvällisen vertailevan analyysin aktivointitekniikoista, joita käytetään RAS-systeemin biosuodatuksessa. Lisäksi tämä on ensimmäinen tutkimus, joka analysoi kuinka aktivointitekniikat vaikuttavat biosuodattajien aktiivisuuteen sekä heterotrofisten ja nitrifikoivien bakteerien suhteellisiin osuuksiin biofilmissä. Analyysi on tehty hyödyntämällä hiilen ja typen stabiileja isotooppeja.

Näiden kysymysten tarkastelua varten suoritin tutkimuksen Suomen Luonnonvarakeskuksessa Laukaalla, Suomessa. Meillä oli kahdeksan kokeellista RAS-yksikköä ja neljä erilaista testattua käynnistysmenetelmää (kylmäkäynnistys-menetelmä kalan, ammoniakin, ammoniakin + typen, ammoniakin + typen + glukoosin kanssa), jotka toistettiin kahdesti.

Tutkimus oli jaettu kahteen vaiheeseen. Aktivointivaihe kesti viisi viikkoa (10.2. – 16.3. 2016), minkä jälkeen kirjolohet (keskimäärin 0,6 kg, biomassa 20 kg tankkia kohden) istutettiin yksiköihin. Istutuksen jälkeen kaikki muut lisäykset lopetettiin. Kaloja ruokittiin ympäri vuorokauden 1,6% tasolla päivää kohden. Kaikissa kokeissa kalat lisättiin 25 päivää biosuodatuksen aktivoinnin jälkeen.

Aktivointivaiheessa ammoniakkitasot olivat korkeimmillaan. Kaikissa muissa paitsi glukoosilla suoritetussa kokeessa, kalojen lisäyksen jälkeen nitraattipitoisuudet kasvoivat, mikä

signaloi nitrifikaation aktivoitumisesta. Korkein nitraatti 3:n pitoisuus oli kylmäkäynnistyksessä, jota seurasi lähellä peräss ammoniakin sekä ammoniakin ja typen kokeet. Glukoosilla tehdyssa kokeessa ammoniakkitasot jatkoivat kasvamista,mikä signaloi matalaa nitrifikaation aktiivisuutta. Aktivointijakson aikana kylmäkäynnistyksessä ja glukoosikokeessa biofilmin hiilipitoisuus oli jo valmiiksi korkea, kun taas ammoniakin sekä ammoniakin ja typen kanssa tehdyissä kokeissa hiilipitoisuus nousi nopesti kirjolohien lisäyksen jälkeen, mikä osoittaa biofilmin biomassan kehittymistä. Biofilmin δ13C heijasti ulosteen isotooppiarvoa ja DICiä muissa paitsi glukoosikokeessa, jossa δ 13C oli lähtöisin heterotrofissa käytetystä glukoosista. Biofilmin δ13C korreloi positiivisesti typpipitoisuuksien kanssa, korkeimman δ13C arvon löytyessä ammoniakin, ammoniakin ja typen sekä kylmäkäynnistyksen kokeissa, mikä viittaa niiden korkeaan nitrifikaation potentiaaliin. Glukoosikokeen biofilmin δ13C heijasti pääasiassa orgaanista heterotrofista uudelleenmineralisoitumista.

Kylmäkäynnistys-kokeilu on demonstroitu yhtä tehokkaaksi menetelmäksi kuin kemiallinen käynnistys. Tulokset viittaavat, että kylmäkäynnistys 1) kasvattaa biofilmin nitrifikaatiotasoa biosuodatussysteemissä muiden testattujen kokeiden tapaan ja 2) parantaa maturaatioaikaa yhtä tehokkaasti kuin kemikaaliset käsittelyt. Tulokset saattavat yllättää niitä, jotka ovat uskoneet kemiaalisten käsittelyiden valloittamattomaan erinomaisuuteen. Myös glukoosiavusteinen menetelmä johti heikkoon nitrifikaation aktivoitumiseen, mikä osoittaa glukoosin lisäämisen kasvattavan heterotrofisten bakteerien määrää biofilmissä. Tällöin nitrifikaatiobakteerit ovat tehottomampia nitrofikaatiossa. Yhdistämällä laboratorio- ja kenttätyön stabiilien isotooppien analysointiin on mahdollista saavuttaa täsmällisiä ja yksityiskohtaisia tuloksia nitrifikaation aktiivisuustasoista biosuodatus-menetelmissä.

Contents

1. INTRODUCTION

One introduction in recent decades in the aquaculture sector is the recirculating aquaculture system (RAS). These systems conserve water by reusing it and treating it to preserve good water quality for the species that is being aquacultured (Figure 1). One of the key components of a RAS is it's bio-filtration system, which is utilized to transform the ammonia that is released from the fish into the non-toxic form of nitrate. In general, nitrification is a two-step process (Timmons and Ebling 2010). Bacteria of the genera *Nitrosomonas, Nitrosococcus*, *Nitrosospira*, *Nitrosolobus,* and *Nitrosovibrio,* participate in the first step of nitrification, oxidation of ammonia to nitrite, whereas the second step, oxidation of nitrite to nitrate involves the genera *Nitrobacter, Nitrococcus, Nitrospira*, and *Nitrospina* (Hagopian and Riley 1998). Nitrifying bacteria are chemoautotrophs, which is a form of chemolithotrophy, use inorganic compounds for ATP synthesis (Timmons and Ebling 2010).
The ideal bio-filtration system would,

> "maximize media specific surface area and remove all of the inlet ammonia concentration, generate little nitrite, maximize oxygen transfer, require a relatively small footprint, use inexpensive media, have minimal head loss, require very little maintenance to operate, and would not capture solids." (Timmons and Ebling 2010)

To achieve all of these parameters there are many different methods that have been devised, each with their own advantages and disadvantages. The average surface specific TAN removal in the fixed bed bio-filtration systems was significantly higher than in the moving bed bio-filtration system (0.20 vs. 0.14; g $N/m^2/d$). (Pedersen *et al.* 2015). A fixed or moving bed bio-filtration systems are the most stable bio-filter methods (Timmons and Ebling 2010). Because of their stability, more RASs have fixed and moving bed systems, systems where the bacteria cover a filter media and through the process of diffusion the dissolved nutrients and oxygen are available to the biofilm (Timmons and Ebling 2010). The disadvantage of a fixed bed system is that they can be quickly overcome with the introduction of heterotrophic bacteria.

In addition to ammonium, fish bring organic matter to the system (faeces and feed), which can favor heterotrophic bacteria, which could then outcompete nitrifiers. In a system that is active and has fish there is an additional parameter of the organics that are released through excretion. If these organics have a level that is too high for the system the nitrifying bacteria in the bio-filter will be outcompeted, and the nitrification process will be less active (Ohashi *et al.* 1995, Chen *et al.* 2006, Michaud *et al.* 2014).

The usage of stable isotopes in a recirculating aquaculture system is a new and novel approach. In the only study that has been previously utilized stable isotopes is the RAS environment, the effects of water filtration on microbial nitrogen cycling was studied by tracing changes in natural isotopic signature of nitrate (Holl *et al.* 2011).

2. LITERATURE REVIEW

2.1. Recirculating Aquaculture Systems

Fisheries products are the last mass marketed food that is being supplied to consumers by "hunter gatherers" (Timmons and Ebling 2010). Due to the fact that we have reached capacity of the catch from waters around the world, aquaculture is the fastest growing segment of agriculture, and is projected to continue to increase at an annual rate of 2.8% per year (Table 1;

Lucas and Southgate 2012). One introduction in recent decades in the aquaculture sector are the Recirculating Aquaculture Systems (RASs). These systems conserve water by reusing it and filtering it to preserve good water quality for the species that is being aquacultured. RASs surpass conventional aquaculture methods, such as outdoor pond systems and net pen systems because they are more sustainable in the long term (Timmons and Ebling 2010).

Table 1. Contributions from Wild Catch and Aquaculture (Timmons and Ebling 2010)

Production (Million ton)	**1950**	**1960**	**1970**	**1980**	**1990**	**2000**	**2007**	**2020 estimated**
Wild Catch	19.2	34.7	63.7	68.2	85.9	96.8	99.3	129.8
Aquaculture	0.6	2.0	3.5	7.3	16.8	45.7	55.4	103.2
Total	19.8	36.7	67.2	75.5	102.7	142.5	154.7	223.0
% from Aquaculture	3	5	5	10	16	32	36	44
World Population (billions)	2.556	3.04	3.709	4.453	5.283	6.082	6.670	7.202
Per Capita Food Fish Supply, kg	5.2	8.0	12.1	11.3	12.9	15.6	17.0	17.1

A RAS has many beneficial traits that the traditional aquaculture methods lack. There is minimal chance of disease introduction in a RAS, unless already diseased fish are introduced to the system. Also, if there is a disease outbreak, the outcome is more manageable than with traditional outdoor system (Timmons and Ebling 2010). This is due to the fact that the tanks are generally smaller per volume size but larger in quantity so if a disease enters only those diseased tanks would have to be dealt with. Apart from this, traditional methods have difficulties controlling the growing cycles, which leaves peaks and troughs in the market supply. In a RAS, the tanks are staggered so that the fish reach production age at different times so that to disperse the stock as it enters the market. Also, there is no chance of escapees entering the wild environment in a RAS (Timmons and Ebling 2010).

RAS technology has steadily developed over the past 30 years and is widely used for brood-stock management, in hatcheries, and increasingly for salmon smolt production around the world (Murray *et al.* 2014). However, the progress of using a RAS for grow-out to market size products has been more restricted. The reasons for this include challenges of economic viability and operating systems at commercial scale (Murray *et al.* 2014).

RAS-designed aquaculture systems are infinitely scalable though. This is due to the fact that there are no environmental limitations to the size (Timmons and Ebling 2010). The basic design of a RAS operates by filtering water from the fish (or shellfish) tanks so it can be reused within the tank. This dramatically reduces the amount of water and space required to intensively produce seafood products. Simplified, the steps in RAS include solids removal, ammonia removal (through nitrification and denitrification), CO_2 removal and oxygenation (Figure 1). The first step of an RAS is usually solid removal through particle filtration, which removes the organic carbon and phosphorous that originates from the faeces and uneaten feed. All parts of the RAS contribute to the function of the whole. However, one essential part is the way of controlling the levels of nitrogen (N) in the system. One important step in removing the nitrogen is, transforming it from the toxic ammonia, into nitrate, a non-toxic form of nitrogen. This

process is done in the bio-filtration system and is called nitrification (Timmons and Ebling 2010). After nitrification, the denitrification process can occur, removing the nitrate from the system entirely through denitrifying bacteria oxidizing it to N_2 gas. After this, aeration removes the CO_2 and adds O_2 to the system. This is usually followed by oxygenation and some form of disinfection, whether it is ozone or ultraviolet light.

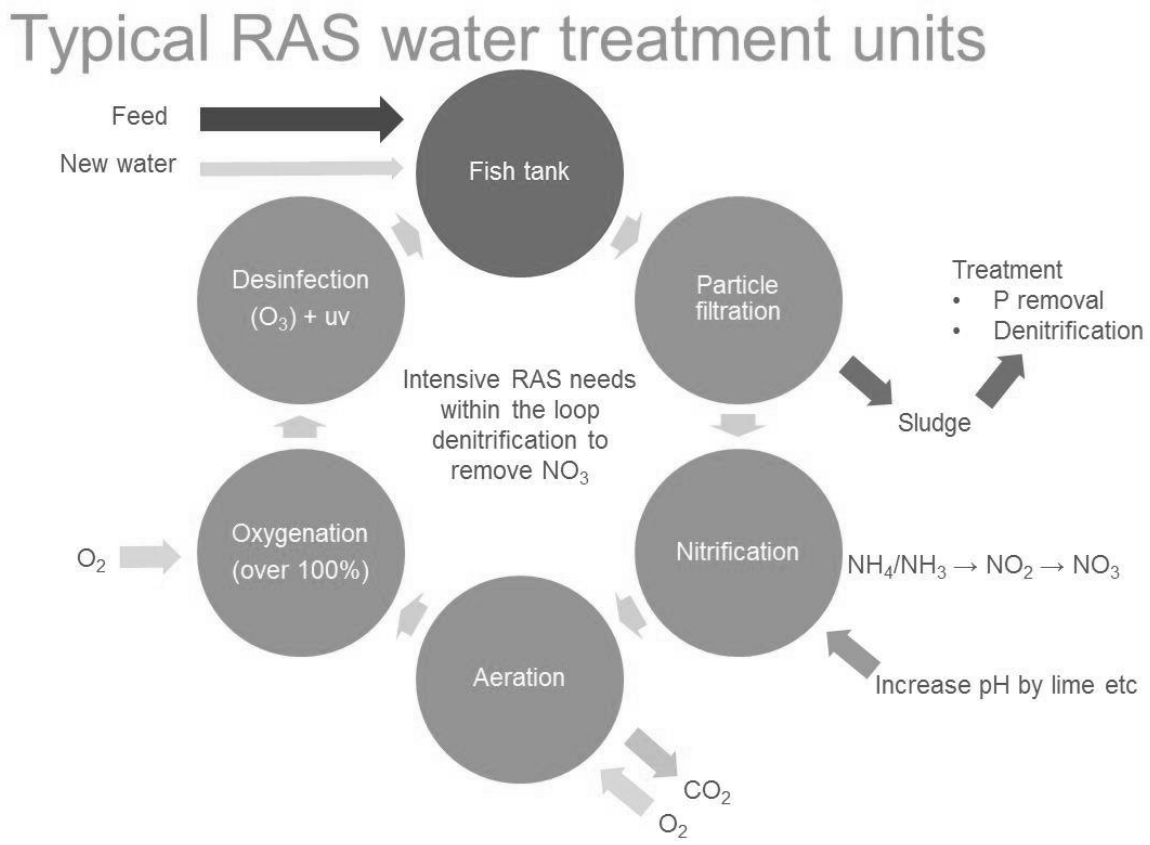

Figure 1. Typical Recirculating Aquaculture System (RAS) water treatment units (copyright: Jouni Vielma)

2.1.2. Nitrogen in a RAS

N is an essential nutrient for all organisms, being present in the form of proteins, nucleic acids, adenosine phosphates, pyridine nucleotides, and pigments (Hagopian and Riley 1998). Fish excrete various nitrogenous wastes through gill diffusion, gill cation exchange, urine and feces. In a RAS system, uneaten feed also contributes to the levels of nitrogenous waste loading. For salmonids the overall maximum retention of N in the body of the fish was found to be 40-50% so the fish excretes the majority of the nitrogen without it being retained (NRC 2011).

In RAS, the main challenge concerning nitrogen is to remove ammonia from the system. Ammonia, which is the main form of N excreted by aquatic species, is a small and highly lipid-soluble molecule and readily eliminated from the blood by branchial diffusion (Hagopian and Riley 1998). Ammonia and ammonium excreted from the gills of aquacultured fish comprise between 60–90% of all excreted N by the fish (Hagopian and Riley 1998). Ammonia exists in

aqueous solution in two forms: NH_3 and NH_4^+. The proportion that these solutions exist depends on the pH (Timmons and Ebling 2010). Although both forms may be toxic to fish, unionized ammonia (NH_3) is the more toxic form at low concentrations (Chen *et al.* 2006). Total ammonia nitrogen (TAN) concentration is used as the key limiting water quality parameter instead of ammonia nitrogen in intensive aquaculture systems due to variations of ionized and unionized ammonia at different pH and temperatures (Chen *et al.* 2006). Urea is also largely expelled through the gills and accounts for 9–27% of the soluble N excreted (Clark *et al.* 1985). Unlike most vertebrates >80% of nitrogenous wastes are excreted by the gills, with only trace amounts excreted by the kidney as urine (Farrell 2011).

Rainbow trout, *Oncorhynchus mykiss*, feed and faeces contain approximately 6 and 3-4% N of their dryweight, respectively (Penczak *et al.* 1982). Also the dietary phosphorus (P) contents for diets without and with soy-derived proteins are 10.5 and 6.9 g P kg^{-1} respectively (Vielma, et al. 2010). Both unionized ammonia and nitrite (NO_2^-) are toxic to fish at low concentrations. Acute toxicity can occur at 0.2 mg/L NH_3 in salmonids and a maximum permissible level of just 0.002 mg/L NH_3 (Hagopian and Riley 1998). To protect fish under most conditions, the recommended level of nitrite (as NO_2-) in soft water is < 0.1 mg/L (Timmons and Ebling 2010). At a nitrate level of 80–100 mg/L, juvenile rainbow trout have found to show signs of toxicity e.g. swimming sideways in the water (Davidson *et al.* 2014). In general, if there is no denitrification, the nitrate level in RAS depends on the make-up water volume (Liters per kg feed typically 200-1000). In a sample of working RASs, the average TAN was found to be 2.4mg/L, and according to the farmers, this concentration did not cause any problems to the fish (Dalsgaard *et al.* 2013). In the same survey, the average NO_2 concentration was found to be 0.15 mg/L and average NO_3 concentration 7.2 mg/L, both well below the lethal level. Overall, nitrification is an essential step of any RAS and can be one of the most challenging and time consuming steps in maintenance of a RAS.

2.2. Biofilters in RAS

In general, nitrification is a two-step process (Timmons and Ebling 2010), the transformation of ammonia to nitrite as well as the proceding step the changing of nitrite to nitrate. In the bio-filtration systems, bacteria are attached to the surface of the biomedia as a biofilm, which is a thin but robust layer of mucilage containing a community of bacteria and other microorganisms (Chen *et al.* 2006). Nitrification has been found to be restricted to a narrow zone of 50 μm of the very top of the biofilm (Dworkin *et al.* 2006, Michaud *et al.* 2014). The distribution of nitrifying bacteria is not equal for the different genera: the oxic part of the biofilm has found to be dominated by ammonia oxidizers e.g. by members of the genus *Nitrosomonas (*(Dworkin *et al.* 2006, Schramm et al. 2000), whereas nitrite oxidizers, e.g. genus *Nitrospira* sp., are most abundant at the oxic-anoxic interface. In the anoxic part of the biofilm, the cell numbers of all the nitrifers have found to be relatively low (Dworkin et al. 2006).

Although the idea of biofilters is to maintain low levels of (TAN), the minimum TAN concentrations have to be high enough to support an activated biofilm. If TAN load is not high enough, bacteria will not survive. Zhu and Chen (1999) evaluated the minimum TAN concentration for submerged nitrification biofilter system, and found mean value of minimum TAN concentration being 0.07 ± 0.05 mg L^{-1} at 27.2 °C. Fortunately to aquaculturists, the minimum TAN concentration are much lower than the toxic level for almost all the aquacultural species (Chen *et al.* 2006).

The efficiency of biofilter system can be controlled by several factors. As nitrification is aerobic process, oxygen is the most important factor in controlling nitrification, and efficient circulation is needed to get oxygen to all parts of the biofilm. The growth rate of ammonia oxidizing *Nitrosomonas* has found to be independent of the dissolved oxygen (DO) concentration above 1.0 mg L^{-1}, while the growth rate of nitrite oxidizing *Nitrobacter* has found to be independent only above 2.0 mg L^{-1} of DO (Stenstrom 1980), suggesting that to cultivate a biofilm that is capable of performing nitrification efficiently, DO should not decrease below 1.0 mg L^{-1}. In addition, water temperature can affect nitrification: higher temperature speeds up the activities of the nitrifying bacteria (Chen *et al.* 2006). However, the impacts of temperature on nitrification are usually very slight: .with no oxygen limitation, temperatures from 14 to 27ºC were not found to affect on the nitrification rate of a fixed film bioreactor (Timmons and Ebling 2010)

The time of feeding is also important in controlling the nitrification activity of the bio-filtration system. If the feeding regime is high at a particular time without having a periodic gradual feeding, it is possible that a pulse in the excretion of TAN will occur (Dalsgaard *et al.* 2015). These higher levels of TAN may exceed the capacity of the biofilter system, resulting in poor water quality in the RAS (Dalsgaard *et al.* 2015). The feeding rate has not been found to control the efficiency of the bio-filtration system, as the efficiency of biofilter system degrade ammonia, urea, and nitrite was found to be similar under different feeding loads (von Ahnen *et al.* 2015).

In a RAS study conducted with Atlantic Salmon smolt (*Salmo salar),* the total aerial nitrification levels was found to be highest at the lowest alkalinity (Summerfelt *et al.* 2015). However, the recommended medium alkalinity level is 70 mg/L, due to the relatively low loss of inorganic carbon compared to highest alkalinity level (Summerfelt *et al.* 2015). The increaced pH stability and reduced TAN concentration are also reasons to choose the medium alkalinity level.

If carbon coming from faeces and uneaten feed is not collected efficiently, the C:N will rise, leading to decreased proportion of nitrifers in the biofilters (Ohashi *et al.* 1995, Chen *et al.* 2006, Michaud *et al.* 2014). This is due to the nitrifiers being outcompeted with the faster replicating heterotrophic bacteria, which utilize organic carbon as their energy source. Under steady state conditions, glucose addition (sucrose) has been found to affect the nitrification rates of bio-filter (Chen *et al.* 2006): a C:N ratio of 1.0 to 2.0 (mass of organic C to mass of N) led to 70% reduction of TAN removal rate as compared to C:N ratio of 0. To keep the nitrification rate high, it is important to reduce, and ideally, to remove the organics from RAS system.

2.2.2. Bio-filter Activation Methods

The activation phase of biofilter is a start up process. First, the ammonia oxidizing bacteria (e.g. *Nitrosomonas*) act, leading to levels of ammonia drop off and the nitrite levels begin to rise, usually approximately two weeks after the startup. Then, after a month, the nitrite levels begin to drop, as slower growing nitrite oxidizers (e.g. *Nitrobacter*) start to transform the nitrite into nitrate, where after the nitrate levels increase and the nitrite and ammonia levels stabilize. The overall length of the activation process is influenced by several factors e.g., temperature, salinity and pH (Timmons and Ebling 2010).

The benefits of seeding the RASs before introduction of culture species are various. One important attribute is that it reduces the stress on the newly introduced stock. Also, it allows higher feed rates from the first day of stocking, increasing the growth rate of fish, which is

important to the economics of a RAS. Finally, it creates a better water quality, improving health and survival of the cultured species (DeLong and Losordo 2012). This is because the ammonia levels should be low at fish introduction, allowing higher feeding rate and also higher stocking levels.

The first method to start up a new RAS is an inoculation. This method includes taking biofilm or sludge from an already activated system and using the ammonia and nitrite that is already present in the activated system to grow the bacteria in the new unit, using this method requires that RAS is already functional. This method usually surpasses another method called "the cold start" (Kuhn *et al.*2010), which means introducing the fish to an un-activated system and slowly building up the levels of bacteria from the introduced fish and also from the make-up water. Another method is the introduction of chemical ammonia. In this method, a sample of bacteria from an already activated biofilter will still have to be used for the initial start-up, but in addition ammonium hydroxide, or unscented household ammonia, will be used to raise the total ammonia level to between 3 and 5 mg/L (DeLong and Losordo 2012). This method has an advantage over the inoculation method due to the lower chance of diseases and pathogens transferring to the new system (DeLong and Losordo 2012). Also, instead of adding ammonia it is possible to also add nitrite to the system in the form of sodium nitrite ($NaNO_2$). Also it is possible to naturally seed in the same way as adding ammonia as there is probably nitrite in the system that is being inoculated from.

2.3. Stable Isotope Research and stable isotopes in RASs

The usage of stable isotope analysis (SIA) for bio-filtration system research is a relatively new approach. Of the 92 elements 71 occur in different isotopic forms (Meier-Augenstein 2010). The vast majority of these are stable isotopes, which are isotopes that do not decay. An isotope shares the same position in the periodic table as its corresponding element, because it has the same number of electrons and protons. However, it has a different number of neutrons. The negatively charged electrons react to form bonds between the atoms (Fry 2006). Stable isotope analysis is a method of analyzing the composition of elements and the ratios of their isotopic signatures.

Two of the most utilized isotopes are nitrogen 15 ($\delta^{15}N$) and carbon 13 ($\delta^{13}C$). For both of these isotopes, the low mass i.e. "light" isotope is by far the most abundant (>95%; (Fry 2006). These two isotopes are used frequently for ecological studies, especially for studying the origins of organic matter in the biosphere (Fry 2006).

Stable isotope of nitrogen ($\delta^{15}N$) is a common indicator for an organism's diet, trophic level and subsistence. $\delta^{15}N$ can be used to determine different N transformation processes and sources in a biofilter system. For example, in a wastewater treatment plant, where nitrifying biofilm community was destroyed after a large storm, the connections between the recovery rate of the biofilter and the relative abundance of total N coming from up river were examined by measuring natural abundance of nitrogen stable isotopes. It was concluded that the rate of recovery for the biofilm was largely based on the availability of N entering the system. (Merbt *et al.* 2011).

There are not many isotope studies from RAS. However in one, conducted in a RAS with Pacific White Shrimp (*Litopenaeus vannamei)*, the effects of water filtration on microbial nitrogen cycling was traced using natural nitrogen stable isotope analysis (Holl *et al.* 2011). In the study, the main aim was to examine whether a foam fractionator or a propeller-washed bead filter would be more effective at eliminating the organic solids from the system and promoting the nitrification rate. Although it was found out that both treatments reached nitrate accumulation

stage at approximately the same time, stable isotope analyses revealed that the microbial community responded differently to the filtration technique and filter systems had distinct dominant N cycle pathways.

Another study utilizing RASs looked into rearing, *Farfantepenaeus paulensis* juveniles (Ballester, et al. 2010). They were reared in a suspended microbial flocs system and fed practical diets containing increasing amounts of crude protein (250, 300, 350, 400 and 450 g kg^{-1}CP) (Ballester, et al. 2010). It was shown that the after 45 days mean shrimp survival rate in the RAS was 89% with no significant difference between treatments. The shrimp that were fed with 250g kg^{-1} or more had a higher weight (Ballester, et al. 2010). A RAS system allows for shrimp culture without compromising the surrounding environment and shows the possible reduction of production costs and fish meal dependence (Ballester, et al. 2010). It was determined through the usage of stable isotope analysis that the microbial biofilm composed by nematodes, diatoms, filamentous cyanobacteria and ciliate may contribute to around 49% of the carbon and 70% of the nitrogen responsibe for *F. Paulensis* juvenile growth (Ballester, et al. 2010). In this study only a small number of diatoms and nematodes, considered important nutritional sources for shirmp, were found in the floc community, thus showing that there is importase of improving the nutritional quality of the flocs, possibly increasing the performace of the shrimp (Ballester, et al. 2010).

Using biofloc technology in a RAS, another study attempted to analyze the nitrogen and phosphorous dynamics in the production of Pacific White Shrimp, *Litopenaeus vannamei* (Silva and Abreu 2013). They found, using δ^{15}N stable isotope analysis that although the shrimp absorbed 39.1 and 35.0% of the total N and P inputs to the system, the dominant process of ammonium immobilization in the system was oxidation by nitrifying bacteria (Silva and Abreu 2013). The leftover N present in the tanks were from organic sources, with continuous accumulation of nitrogen and phosphorous throughout the experiment. In this biofloc system the removal of these nutrients is important to further consider because they may enhance the growth of harmful algae in tanks and in water bodies that collect the post-crop wastewater (Silva and Abreu 2013). Stable isotope analysis was not used in this study however water samples were taken to observe TAN levels and Chl-*a* levels were determined fluorometrically using a calibrated Turner TD700 fluorometer.

At off-shore fish farms there was a usage of stable carbón and nitrogen isotope ratios to measure the sedimentary organic matter (SOM) collected from 41 stations in and around a coastal fish farm in Japan to quantify aquaculture-derived organic matter in the sediment (Yokohama, et al. 2006). SOM within 30 m from the cages is characterized by its reduced δ^{13}C and enriched δ^{15}N values, reflecting the deposition from the plant and fish-derived elements from the farm, respectively (Yokohama, et al. 2006). As the distance increaced away from the cages the aquaculture-derived organic matter decreased exponentially, with the waste dispersal reach an area up to 300 m (Yokohama, et.al. 2006).

Focusing on gilhead seabream (*Sparus aurata*) a study was interested in using stable carbon and nitrogen isotopes to analyze the composition of the isotopes in the closed system and how it interacted with the fish (Schneider, et al. 2011). The water quality maintenance in the system was based on two biofiltration steps; an aerobic trickling filter in which ammonia is oxidized to nitrate using nitrification and an anaerobic fluidized bed reactor where excess organic matter and nitrate are removed. The δ^{13}C and δ^{15}N of organic matter in the mariculture system indicated that the fish fed only on the feed pellets and not on particulate organic matter. (Schneider, et al. 2011).

3. AIMS

The objectives of this study were to evaluate how different activation techniques affect the 1) overall function, 2) the nitrification activity of the bio-filtration units and 3) the interactions between heterotrophic and nitrifying bacteria in the RAS.

4. MATERIALS AND METHODS

4.1 Study site and design

At the site eight experimental fresh water RAS units (total water volume 1200 liters, tank 500 liters), which each had own separate water treatment and water quality measurement systems were utilized. The water treatment included a swirl separator, drum filter, four separate bio-filtration chambers (normally two moving bed and two fixed bed filters), packed column, oxygenation, and pH-control. In the packed column, water was trickled through bioblocks against air current. Air blower was portable adjustable, based on continuous CO_2-measurement. Water circulation in the system was controlled by variable speed pumps. Replacement water was added by dosing pumps, accurate at small water replenishment rates. Each system had an optical probe to measure nitrite, nitrate, total organic carbon and turbidity. In addition, optical oxygen probes, CO_2-probes and pH sensors were included. During the experiment, the temperature was set between 12-15ºC and roughly 1-2% of the circulating in the RAS was new daily.

I used four different start-up treatments: cold start (fish biomass and feeding, no chemicals added in activation), ammonia treatment (NH_4 added, with additions occurring every one to three days of 11-16g NH_4Cl) (Table 2), ammonia and nitrite treatment (NH_4 + NO_2, roughly 17-21g added nitrite and 11-16g NH_4Cl every few days (Table 2.), and ammonia, nitrite and glucose (NH_4, NO_2 + Glc, with a ratio between C and N of 1:1, 40g glucose was added every three days) (Table 2.), two replicate pools per treatment. The experiment was divided to two phases. The activation phase continued for five weeks (Feb 10 - Mar 16, 2016) after which rainbow trout (average size appr. 0.6 kg, biomass 20 kg per tank) where added to the units. Once the fish were added all of the additions in the treatments were stopped. Fish were fed 24 hours per day, at the rate of 1.6% per day. The addition of the fish in all the treatments after activation of the biofilter system was measured for a period of 25 days.

Table 2. Ammonia, Nitrite, and Glucose additions amount and time

Tank	10	10	10	9	9	8	5	5	5	4	4	2
date /Chemical	NaNO2 (g)	NH4Cl (g)	Glucose (g)	NaNO2 (g)	NH4Cl (g)	NH4Cl (g)	NaNO2 (g)	NH4Cl (g)	Glucose (g)	NaNO2 (g)	NH4Cl (g)	NH4Cl (g)
11.2.	17.2	13.4		17.2	13.4	13.4	17.2	13.4		17.2	13.4	13.4
12.2.	17.2	13.4	40	17.2	13.4	13.4	17.2	13.4	40	17.2	13.4	13.4
15.2.	21.9	16.2	40	20.7	14.7	15.1	21.1	15.4	40	20.6	15	15.3
17.2.	14.2	10.6	40	12.3	10	10	16	10.5	40	13.4	10.2	10.1
19.2.	17.4	12.6	40	15.9	11.9	12.1	16.7	12.5	40	16.6	12.2	12.5
22.2.	19.8	15.3	40	19.8	14.8	14.9	19.3	15.4	40	19.6	14.8	15
24.2.	16.5	12.2	40	15.3	11.1	11.4	15.7	11.7	40	14.8	11.4	11.7
26.2.	19.3	14	40	16.9	13	13.2	20	13.3	40	18.3	13.2	13
29.2.	20.2	14.9	40	18.5	14.7	14.9	21.2	15.5	40	20.5	14.9	15.6
2.3.	15.8	12	40	16.6	11.6	11.5	14.7	11.8	40	15.4	11.5	11.5
4.3.	17.6	11.9	40	17.8	11.5	12.2	17.3	12.3	40	17.4	12.1	12.2
7.3.	19.8	14.9	40	27.1	14.9	15.1	19.3	15.3	40	24.4	14.8	15.5
9.3.	15.8	11.4	40	27.1	10.9	11.8	16.4	11.6	40	27.1	11.6	12.2
11.3.	16.4	12.3	40	27.1	12	12.8	16.5	12.5	40	27.1	12.1	13.5
14.3.	21.1	14.8	40	27.1	14.9	16.7	20.2	15.2	40	27	15.2	17.8
Total amount (g)	270.2	199.9	560	296.6	192.8	198.5	268.8	199.8	560	296.6	195.8	202.7

4.2. Sampling

The physiochemical characteristics of TOC, turbidity, pH, temperature, TSS, nitrite and nitrate were all measured during the experiment using spectrometer (Franatech. S::can spectro::lyser V2.1 spectrometer). Also, a pH Prominent to test pH was used. Ammonia was measured by a spectrophotometer. Each tank had two O_2 systems (optical S::can and galvanic Oxyquard) and CO_2 spectrometer probe (Franatech. S::can spectro::lyser V2.1 spectrometer). Tank covers had built-in LED lights. Water flow measurement by Bürkert and a Hach photometer at the site were also utilized. The RAS technology, including the computers, was developed by AKVA Group. The rates of these physiochemical characteristics in the systems were monitored during the whole experiment to determine how the biofilter system units activated and coped with the introduction of the fish. All of these parameters were measured every six minutes, except ammonia, which was measured once a day. To follow carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) stable isotope values of the biofilm before and after fish addition, samples of the bio-carriers from the biofilm of each treatment were taken before fish addition, and -1, 1, 5, 7, 14 and 21 days after that. For that, I took two samples of fifteen bio-carriers per experimental unit. In addition, samples of the faeces, as well as of feed, were collected to measure baseline values for $\delta^{13}C$ and $\delta^{15}N$. All isotope samples were frozen prior to analyses.

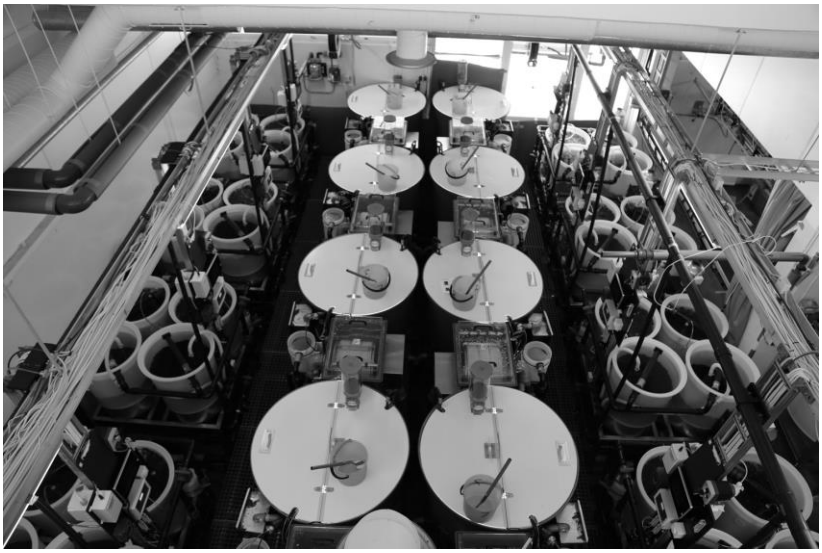

Figure 2. Laukaa Fish Farm Experimental RAS Facilities

4.2.1. Nitrification activity measurements

To measure nitrification activity, two sets of laboratory incubations with 15-labelled ammonium were conducted. First experiment took place after the activation period (March 16th) and the second 25 days after the introduction of fish (April 5th). For the experiments, I collected biocarriers from each moving bed pool and transferred them from Laukaa to University of Jyväskylä. In the laboratory, we divided the biocarriers into experimental vials with 360 ml water from the incoming flow at Laukaa, and 60 ml of 15-labelled ammonium (final concentration of 7.6 mg/L). We had thirty biocarriers per vial and two vials per pool. Incubation occurred from 3 hours in the first experiment to 4.5 hours in the second at approximately 12°C and under constant mixing by magnetic stirring bars. Water samples to measure the stable isotope composition of nitrite and nitrate produced during the incubation were taken at the beginning of the experiment and when the process of nitrification appeared to have been completed when the ammonia was near 0 and nitrate levels seemed highest, at the end of the incubation. The measurements were done by following the ammonium concentrations and nitrate concentrations with probes as well as a Vernier sensor. After the samples had been collected, PhD Sanni Aalto first froze all the samples, then starting in June she converted the nitrate into N_2O by using method by McIlvin and Altabet (2005) and by Miranda et al. (2001). After that, the N_2O isotopic composition was measured with Isoprime100 isotope-ratio mass spectrometer coupled to an Isoprime TraceGas Pre-Concentration Unit.

4.3 Stable isotope analysis of carbon, nitrogen and %C of biofilm

To trace the changing signatures of the carbon and nitrogen isotope values as well as the %C once the fish were introduced to the system, I removed the biofilm from the biocarriers using an ultrasonic bath (6-8 biocarriers in 50ml plastic tube, 2 tubes/pool, five minutes) and collected the biofilm by centrifuging (6000 rpm, 20min), after which I freeze-dried the samples. After that, I weighed them to around 0.6 mg of the dried the biofilm for the isotope analysis, two replicates per pool. In addition, standard series as well as drift standards were then prepared using dried and homogenized fish muscle as an internal laboratory working standard. The isotopic

composition of carbon $\delta^{15}N$ of the biofilm will be then measured with Carlo-Erba Flash 1112 series elemental analyser connected to a DELTAplus Advantage isotope ratio mass spectrometer (Thermo Fisher Scientific Corporation). As glucose $\delta^{13}C$, we used the previously measured – 11.9 ± 0.05 ‰ (Kankaala et al. 2010).

4.4 Statistical Analysis

For the experiments, there were two replicates per biofilter system and two biofilter systems, making four replicates per treatment. However, the biofilm biomass was so low in the beginning of the experiment in some treatments, so I had to exclude the first sampling date from statistical analyses due to low number of replicates. Also, in the growth and feed results as well as the extended parameters, I had only two replicates (two pools), so statistical analyses would be very insensitive.

For all the statistical analysis I used the Statistical Package for the Social Sciences (SPSS, version 21). First, I used Two-way repeated measures ANOVA and pairwise comparisons with LSD procedure for analyzing differences in %C, $\delta^{13}C$ and $\delta^{15}N$ between treatments during the six sampling occasions. In addition, I studied the interactions between nitrate concentration and $\delta^{15}N$ of biofilm using linear regression. To study differences in the nitrification activity between the treatments at the fish introduction and three weeks after the addition of the fish**,** I used Then Kruskal -Wallis test with pairwise comparisons, as normality assumptions were not met.

5. RESULTS

5.1. Water quality in RAS

In the activation period, all treatments performed relatively similar (Fig. 3.). After the fish were added, all treatments besides the treatment with glucose had an increase in the nitrate concentrations, signalling the performance of nitrification. The highest concentration of nitrate was in the cold start treatment, being followed closely by the ammonia and the ammonia and nitrite treatments (Fig. 3.). In the glucose treatment, the ammonia levels continued to rise, signalling poor nitrification activity.

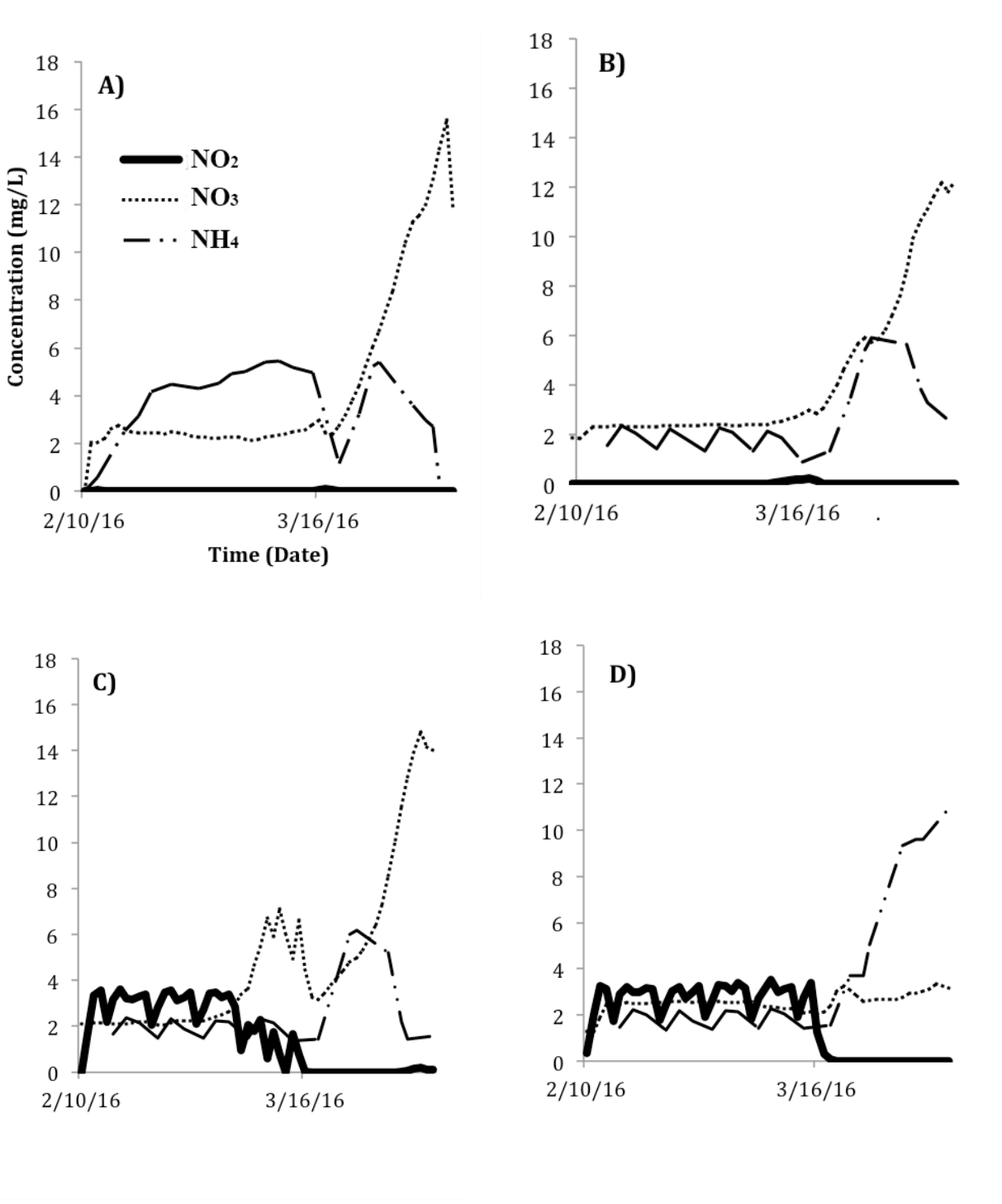

Figure 3. Nitrate (NO_3), Nitrite (NO_2), Ammonia (NH_4) levels in all four treatments from start of activation to 3 weeks after addition of rainbow trout (16th of March), totalling seven weeks. Treatment A has no additional chemicals added and is just the natural incoming water source plus then addition of rainbow trout, Treatment B has added ammonia, Treatment C has ammonia and nitrite added and Treatment D has ammonia, nitrite and glucose added.

5.2. Stable Isotope composition of biofilm

For biofilm %C, no significant interaction between sampling time and treatment was not found, which means that the %C changed similarly in time throughout all the treatments (Two-way repeated measures ANOVA, $F_{4.6,13.9}$= 2.59, P=0.076; Fig. 4). At the beginning, %C was low, and increased in time. However there were differences in %C between treatments ($F_{3,9}$ = 4.51, P = 0.034): %C was significantly higher in the cold start than in other treatments (Pairwise comparisons with LSD, P < 0.05), while no differences between the other three treatments could be observed (P >0.05).

For $\delta^{13}C$, a significant interaction between sampling time and treatment was found (Two-way repeated measures ANOVA, $F_{12,36}$ = 12.17, P < 0.001; Fig. 5), meaning that the effect of treatment on $\delta^{13}C$ varied between sampling times. The main effects could not be interpreted because of this interaction. Within each sampling time, the effect of treatment on $\delta^{13}C$ was studied with pairwise comparisons. On day 1 and 5 days after fish addition, $\delta^{13}C$ of all treatments except ammonium and ammonium and nitrite treatment differed from each other. On 7, 14 and 21 days after fish addition, glucose treatment differed from ammonium and ammonium and nitrite treatments, being more enriched, while the $\delta^{13}C$ of biofilm of all other treatments was found to be similar. The $\delta^{13}C$ of the faeces was -26‰ and the feed was -25.3‰.

For $\delta^{15}N$, a similar interaction between sampling time and treatment was found (Two-way repeated measure ANOVA, $F_{4.66,15.55}$ = 4.060, P = 0.016; Fig. 6), meaning that the effect of treatment on biofilm $\delta^{15}N$ varied between sampling times. Because of this, the effect of treatment on $\delta^{15}N$ was studied with pairwise comparisons within each sampling time. In the glucose treatment, the biofilm had significantly lower $\delta^{15}N$ value than in the other treatments in the beginning (1 and 5 days after fish addition). 7 days after fish addition, glucose treatment had significantly lower $\delta^{15}N$ than ammonium and ammonium + nitrite treatments, but did not differ from the cold start treatment. However, 14 and 21 days after fish addition, the biofilm of glucose treatment had again significantly lower $\delta^{15}N$ than the one in other treatments.

There was a positive relationship between nitrite and nitrate and the $\delta^{15}N$ value (linear regression, $y = 0.5664x + 1.0726$, $R^2 = 0.48$; Fig. 7), indicating that when nitrate levels increased the $\delta^{15}N$ value became more enriched.

5.3 Nitrification Activity Rate

Before fish addition, the nitrification activity differed between activation treatments (Kruskal-Wallis test, H = 12.27, P =0.007; Fig. 8.), as glucose treatment had significantly lower activity than the cold start and ammonium treatments (Pairwise comparisons, Mann-Whitney test, U = 11, P =0.001, U = 8.5, P = 0.012).

After fish addition, the nitrification activity differed between activation treatments (Kruskal-Wallis test, H = 9.09, df = 3, P =0.028), as glucose treatment had significantly lower activity than the other treatments (Pairwise comparisons, Mann-Whitney test, U = 7, P=0.038, U = 7.5, P=0.026, U=9.5, P =0.005; Fig. 8.).

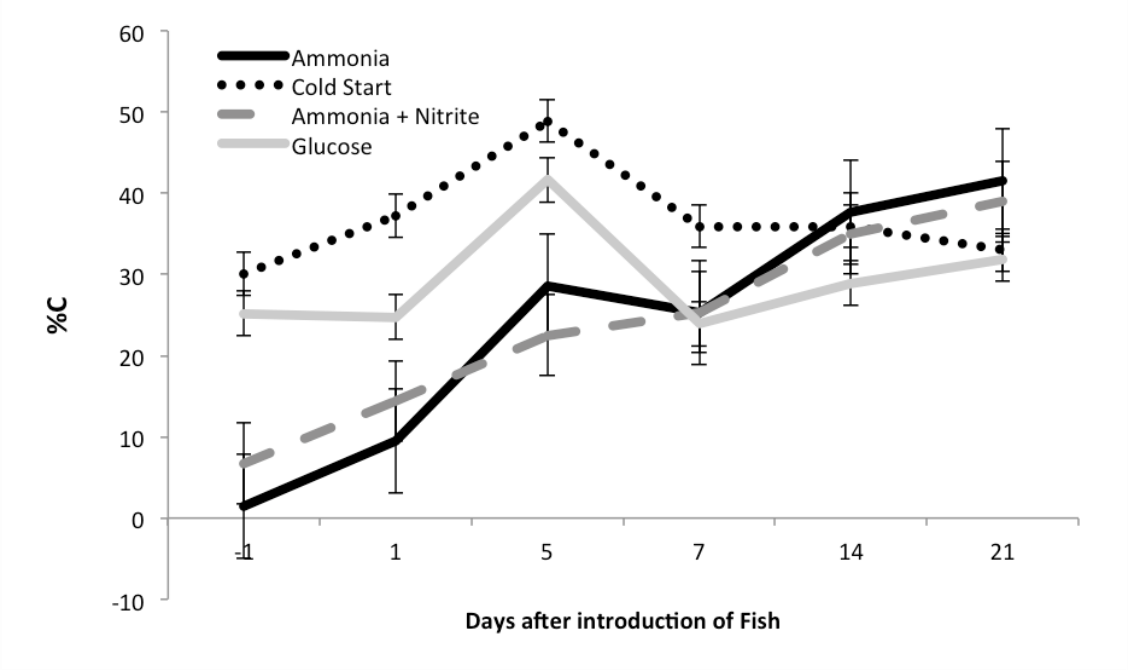

Figure 4. Carbon percentage of the biofilm in the bio-filtration systems in the four treatments

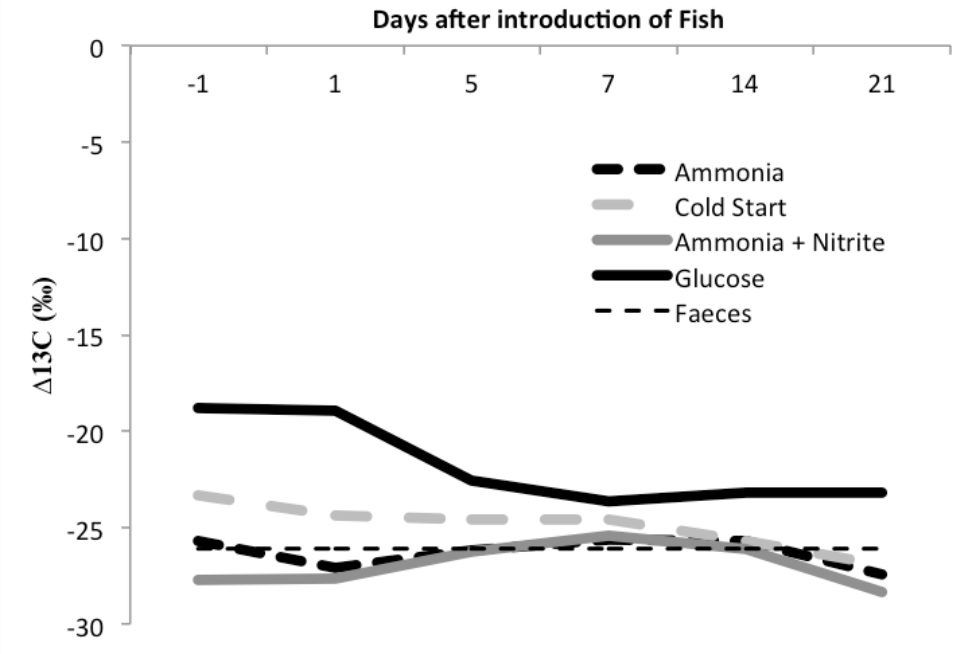

Figure 5. $\delta^{13}C$ values of the four treatments and faeces value. Notice the initial difference of the Glucose treatment

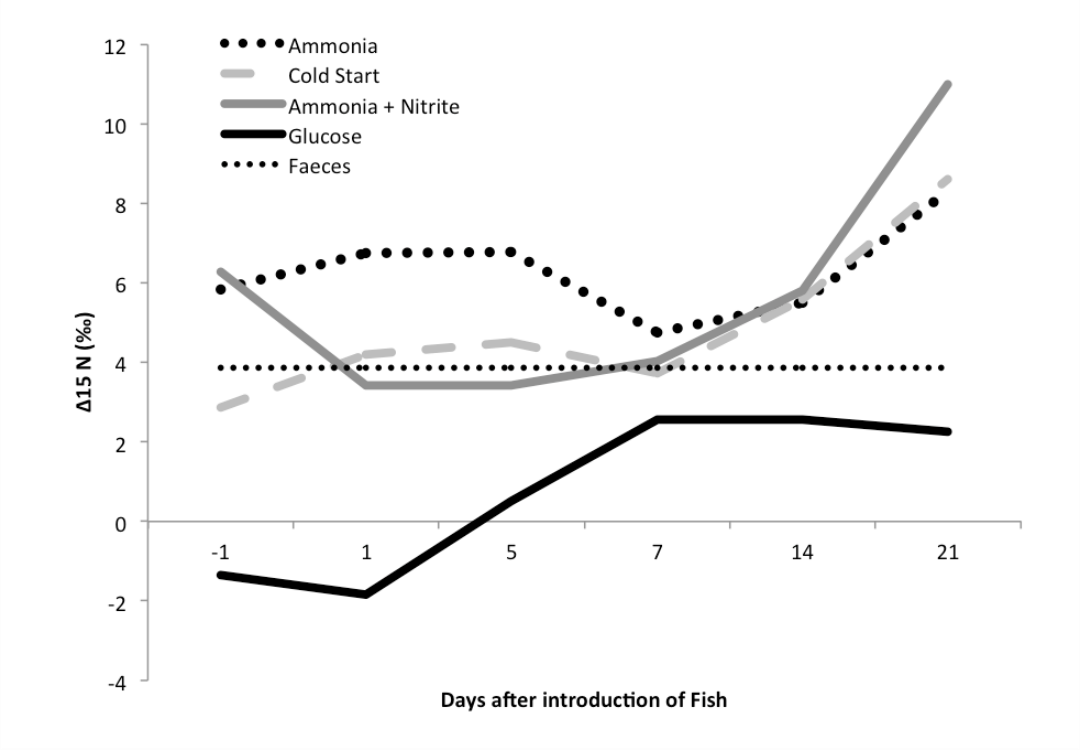

Figure 6. The $\delta^{15}N$ nitrogen value of the biofilm in each treatment

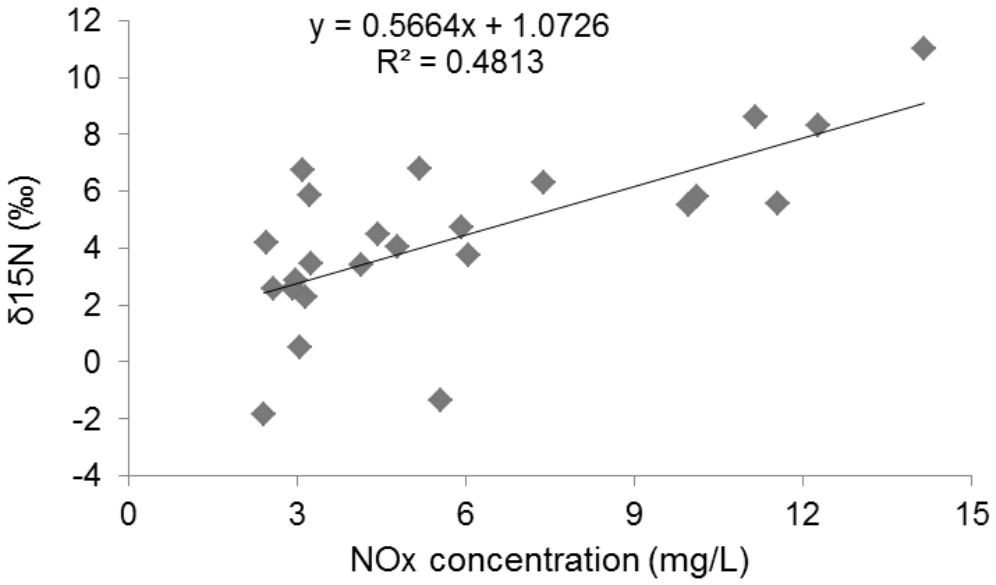

Figure 7. Plot showing positive relationship between nitrite and nitrate and the $\delta^{15}N$ value in all treatments after the introduction of the fish.

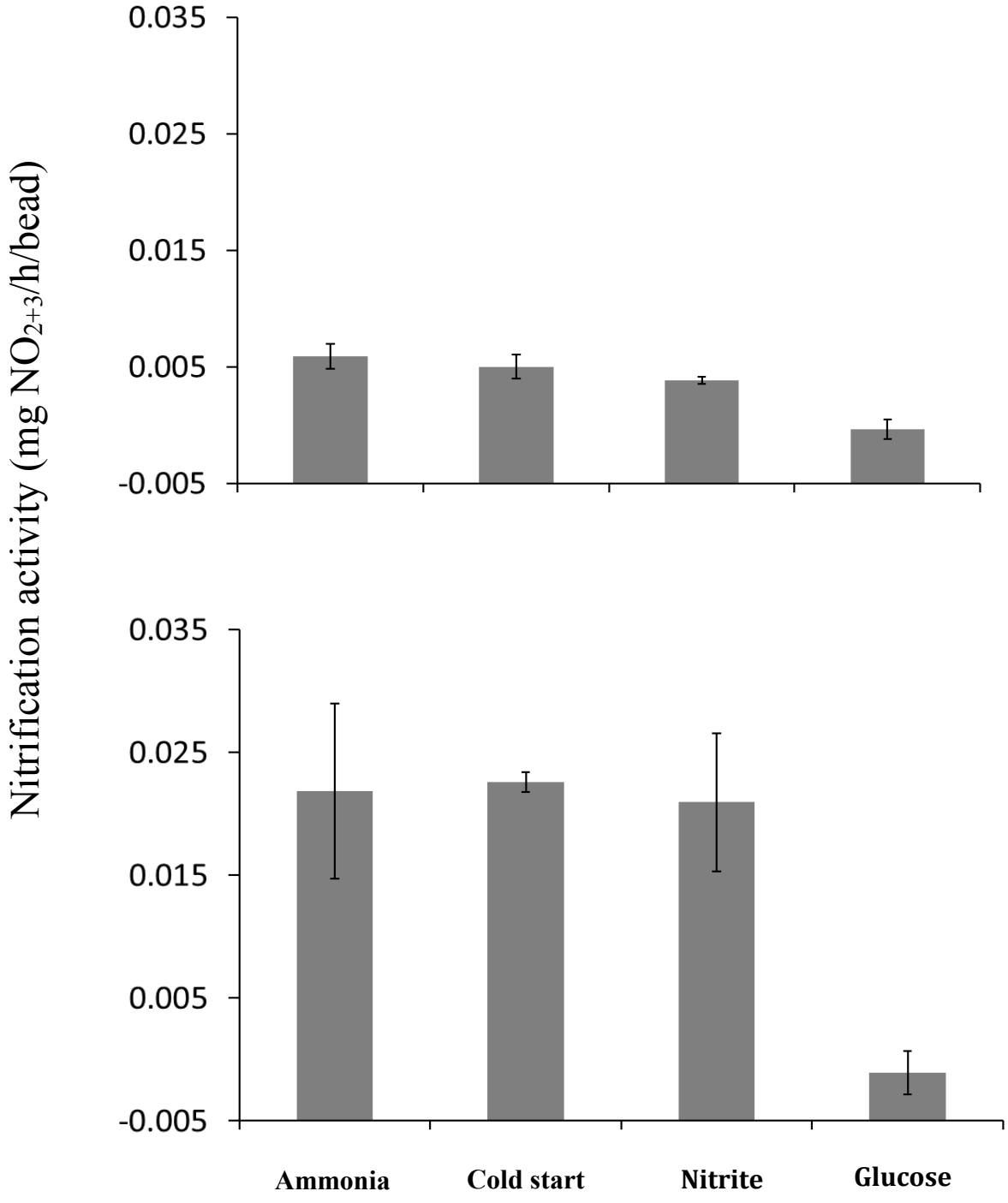

Figure 8. The nitrification activity rates A) after activation was complete and before fish were added and B) three weeks after fish addition. The error bars represent the mean ±. SE. There were two replicates per bio-filtration system and two bio-filtration systems, making four replicate. Ammonia and cold start treatment did not differ from each other, cold start treatment did not differ from Nitrite treatment, and glucose was significantly lower then the other three treatments.

5.4 Experiment Parameters

The growth percent of all the fish in the treatments was around 30% (Table 3) Of the 34 fish; the average starting weight was around 0.55 – 0.6 kg with an ending weight of around 0.75 – 0.8 kg (Table 3.). There was no significant difference in growth of all the treatments. Also many parameters during the introduction of the fish in the Laukaa RAS were taken (Table 4.) (TOC, turbidity, pH, O_2, temperature, TSS, velocity, CO_2, ammonia, nitrite and nitrate levels). These physiochemical measures demonstrate the exact conditions in the treatments and insure that there were no significant differences in the parameters between them. Also they are important for study replication to ascertain significantly comparable results. The CO_2 levels of the chemical treatments, besides glucose started low but all treatments gradually increased to the same signature with the introduction of the fish and higher loading rate. The pH in all the treatments stayed steady at around pH 7 for the entire experiment, except for the glucose treatment, whose glucose levels fluctuated from around pH 4.5 at the beginning to the end at around pH 10 (Table 4.).

Table 3. The growth and feed intake of each experimental unit in the Laukaa experiment

	START				END			GROWTH							
Treatment	n	Mass (kg)	Average (kg)	feeding days	n	Mass (kg)	Average (kg)	Mass (kg)	Average (kg)	Feed intake (kg)	Feed intake kg / d	Feed efficiency	SGR	Feed intake per weight	Growth percent
Ammonia	33	19.3	0.59	25	33.0	25.4	0.77	6.09	0.19	5.58	0.22	0.92	0.81	0.29	0.31
Cold Start	33.5	19.4	0.58	25	33.5	26.1	0.78	6.65	0.19	5.79	0.23	0.87	0.84	0.30	0.34
Ammonia + Nitrite	33.5	19.1	0.57	25	33	25.0	0.76	5.92	0.189	5.66	0.22	0.95	0.85	0.29	0.31
Glucose	31.5	18.9	0.60	25	31.5	24.7	0.78	5.82	0.184	5.52	0.22	0.95	0.78	0.29	0.31

Table 4. The Parameters in the RAS treatments at Laukaa experimental fish farm

COLD START Days after introduction of fish	O_2	CO_2	pH	Velocity	TSS	Temp.	Turbidity	TOC
-1	11.5	7.35	7.27	0.19	11.2	10.5	10.6	8.7
1	11.4	4.66	7.30	0.19	10.3	9.54	10.0	8.06
5	10.6	6.49	7.29	0.19	11.4	9.91	10.6	8.75
7	11.4	7.98	7.16	0.19	12.2	10.0	11.2	9.3
15	11.0	10.8	7.33	0.17	12.6	10.4	11.6	9.77
21	11.7	16.8	7.37	0.17	14.3	10.1	13.3	10.9
AMMONIA Days after introduction of fish	O_2	CO_2	pH	Velocity	TSS	Temp.	Turbidity	TOC
-1	10.4	2.14	6.57	0.196	5.93	10.4	5.58	7.16
1	11.1	6.38	6.52	0.200	5.85	9.69	5.51	7.30
5	10.2	9.54	6.56	0.200	7.52	9.8	7.0	8.92
7	10.7	11.3	6.59	0.194	8.29	9.83	7.79	9.67
15	10.6	14.7	6.6	0.187	6.46	10.06	5.85	8.35
21	10.9	13.0	6.5	0.195	6.0	9.74	5.39	8.23
AMMONIA+ NITRITE Days after introduction of fish	O_2	CO_2	pH	Velocity	TSS	Temp.	Turbidity	TOC
-1	10.2	2.31	7.46	0.198	3.16	10.39	2.62	5.84
1	10.9	6.35	7.34	0.197	2.65	9.57	2.40	5.99
5	9.67	10.5	7.29	0.196	3.57	9.95	3.33	7.16
7	10.5	12.1	7.29	0.194	3.99	9.95	3.71	7.58
15	9.73	13.6	7.33	0.201	2.86	10.15	2.46	6.76
21	10.5	14.9	7.34	0.19	2.96	9.76	2.68	6.98
GLUCOSE Days after introduction of fish	O_2	CO_2	pH	Velocity	TSS	Temp.	Turbidity	TOC
-1	10.3	5.44	4.46	3.84	2.13	2.31	5.50	8.78
1	10.7	7.07	5.80	3.80	2.11	2.29	5.15	8.83
5	10.2	7.31	7.72	3.74	3.04	2.64	5.35	10.1
7	11.0	7.78	8.04	3.74	2.58	2.48	5.32	9.54
15	10.0	9.64	8.89	3.87	2.92	2.84	5.64	10.4
21	10.5	9.65	10.2	3.85	3.23	2.98	5.55	10.8

6. DISCUSSION

Start-up method was found to affect the nitrification process and the overall function of the RASs. During the activation phase, all treatments besides the cold start treatment had total ammonia nitrogen (TAN) levels at around 2mg/L. However, the proportion that NH_3 and NH_4^+ exist depends on the pH in the system (Timmons and Ebling 2010), meaning that lower pH levels will have a higher concentration of NH_4^+, and higher pH levels will have higher concentration of NH_3. The concentration levels are directly proportional to the pH. The pH of the glucose treatment went from low to high meaning that the there was theoretically an accumulation of NH_3 in the treatment, increasing toxicity to the fish. The cold start treatment had highest TAN levels during activation, around 5mg/L, presumably to the increased load in the bio-filter systems from the additional fish. Once the fish were added, there was a dramatic change in the systems, as all treatment pools reached TAN levels of 6mg/L after two days of fish addition. The TAN levels that were the average levels in the average system was 2.4mg/L (Dalsgaard *et al.* 2013), this is substantially lower then the TAN levels in our system after two days of additions. However, quickly all the treatments, except in glucose treatment, TAN levels reduced to around 2mg/L. Compared to the previous survey results ((Dalsgaard *et al.* 2013), the TAN in the Laukaa system was within the normal range of a working RAS, that makes it a good representation for a standardized system TAN concentrations.

One aim of this study was to find whether the addition of carbon potentially mimicking the effects of fish excreted carbon, would promote nitrification rate after the end of the experiment. This is based to the idea that adding carbon to the system might slow down nitrification during activation period, but once the fish are added the biofilms would be more prepared to handle the carbon load that is released from the fish, and thus may perform nitrification more efficiently (Chen *et al.* 2006). However, what was found was that the process of nitrification was greatly hindered in glucose treatment, with ammonia levels rising and nitrate levels staying low, indicating poor nitrification activity and agreeing with earlier studies (Ohashi *et al.* 1995), (Chen *et al.* 2006), and (Michaud *et al.* 2014). This is due to the nitrifiers being outcompeted with the faster replicating heterotrophic bacteria, which utilize the C that is released into the system from the feed and feaces (Chen *et al.* 2006).

Under steady state conditions, sugar addition (sucrose) has been found to affect the nitrification rates of biofilter systems (Chen *et al.* 2006): a C/N ratio of 1.0 to 2.0 (mass of organic C to mass of N) led to 70% reduction of TAN removal rate as compared to C/N ratio of 0. In the Laukaa experiment, the C/N ratio of glucose treatment was 1.0. Based the nitrification activity measurements, the nitrification activity of the cane glucose treatment at three weeks after the introduction of the fish, was roughly 70% of the other activated treatments, supporting the previous findings (Chen *et al.* 2006). In general, this study supports the view that to keep the nitrification rate high it is important to reduce and ideally, remove the organics in a system. However, counter- intuitively, it was found that the cold start treatment performed nitrification as good as the treatments with chemical additions (ammonium and ammonium+nitrite). This seems strange due to having just stated that the less carbon in the system the better, because the fish excrete carbon from their feed (Chen *et al.* 2006). There seems to be a difference from carbon excreted from the fish and an external carbon source in the biofilter systems that fundamentally change the bacterial propagation in the biofilm. It could also be that the fish excrete far more ammonia then carbon which would make the C have much less of an effect on the biofilm.

The idea from a previous study was that the cold start method was inferior to a chemical start-up in the bio-filtration system (Kuhn *et al.* 2010). Our study demonstrates that this is not necessarily true. Our growth percentage of the cold start treatment was highest and the nitrification activity rate was even the same as the chemical start-ups.

The results on stable carbon and nitrogen isotopic composition, as well as the carbon content of biofilm confirmed the differences between the treatments. After fish addition, the carbon content of the biofilter system started to rise, reflecting the increasing carbon load excreted by the fish added. The glucose and cold start treatments had higher initial %C, due to the glucose treatment periodically being seeded with carbon. The cold start treatment had an initial higher carbon level as the excretions from the fish already in the system raise the carbon to levels higher then the biofilter systems that did not have any carbon biomass until the fish were introduced (the chemical added treatments). The %C increase in the system from day 1 demonstrates an accumulation of a heavier load from the excretions of the added rainbow trout. It seems that heterotrophic bacteria in the systems had their own activation that peaked on day 5 and then reached its limit with availability of carbon in the systems. The carbon levels then seemed to settle at around 40% and did not further increase due the systems available carbon from the feaces of the fish; with a heavier feeding rate or more intensive farming the percent carbon would rise. If it rose too high the heterotrophic bacteria could possibly colonize the biofilter systems to an extent that might slow the nitrification process.

After fish addition, the $\delta^{13}C$ values of the four treatments quickly adjusted to the value of the faeces (-26‰), because the dominating source of carbon dioxide rapidly became the faeces or uneaten feed as well as CO_2 respired by the fish. Nitrifying bacteria do not use organic carbon but they use CO_2 as their carbon source. Unfortunately I do not have samples on δ^{13}-CO_2 to measure the main CO_2 source. However, initially the glucose treatment had a higher $\delta^{13}C$ value (-13‰), reflecting the value of glucose (-12‰), which is more enriched than the one of fish faeces. These results paired with the %C increasing in the system to around 40% of the biofilm composition demonstrates the dominance of the new faeces signature excreted from the fish in the system.

The positive relationship between $\delta^{15}N$ of the biofilm and nitrite+nitrate of RAS indicates that when more ammonium was oxidized to nitrite and further to nitrate, the biofilm became more 15-depleted, with the ammonium levels becoming δ^{15} enriched. As the ammonium could be assumed to be almost completely utilized by the biofilter, the biofilm and suspended solids collected together with the biofilm can be considered to reflect mainly the $\delta^{15}N$ of ammonium (Holl et al. 2011), as nitrifiers are assumed to prefer 14-ammonium, leading to enriched ammonium pool. In the glucose treatment, δ^{15} did not enrich after 7 days, unlike in the other treatments. Remineralization of organic matter by heterotrophic bacteria produces NH_4 with a $\delta^{15}N$ equal to the starting material (TSS: $\delta^{15}N$ of faeces = 3.9‰), also there is a possibility that some fractionation might happen having then 15-enriched ammonium produced (Holl *et al.* 2011). The glucose treatment started at $\delta^{15}N$ value of -1‰, and then after day 5 began increasing to the value of the faeces (4‰). The $\delta^{15}N$ of biofilm was more depleted in the glucose treatment, as there was less enriched ammonium due to lower nitrification activity in the biofilter system It seems that no ammonia was used by the biofilm; this is because it was colonized by heterotrophs more than in the other treatments.

7. CONCLUSION

The cold start treatment has been demonstrated to be equally as effective as the chemical startup treatments. Evidence also suggests that the cold start treatment 1) amplifies the nitrification rate of the biofilm in the bio-filtration system, among the tested treatments, and 2) improves maturation time, just as effectively as the chemical startup treatments do. These results may come as a surprise to many who believed in the unassailable superiority of the chemical treatments. As for the glucose-subsidized method, it led to poor nitrification activity, indicating that adding glucose increases the amount of heterotrophic bacteria in the biofilm, which means the nitrifiers are less efficient at nitrification.

Combining lab and field work with stable isotope analysis it is possible to come to accurate and detailed findings about the nitrification activity rates of bio-filtration treatments.

ACKNOWLEDGEMENTS

I want to thank my supervisors Sanni Aalto and Jouni Vielma for enabling me to do this work and introducing me to the fascinating world of recirculating aquaculture and stable isotopes. I want to thank also my international advisor Timo Marjomäki at the University of Jyväskylä for valuable comments in improving my writing. This study was done in accordance with the Natural Resource Institute of Finland (LUKE) and I wish to thank the institute and all personnel involved in the project. Also, I want to thank Prof. Roger Jones and Mikko Kiljunen from the University of Jyväskylä for helping with conceptualization of this project. I am immensely grateful to the entire staff and community of the University of Jyväskylä and affiliated research stations and institutes for their supports throughout my study period.

REFERENCES

Ballester E., Abreu P., Cavalli O., Emerenciano M., De Abreu L. & Wasielesky W. 2010, Effect of practical diets with different protein levels on the performance of *Farfantepenaeus paulensis* juveniles nursed in a zero exchange suspended microbial flocs intensive system. *Aquaculture Nutrition*, 16: 163–172.

Chen S., Ling J. & Blancheton J.-. 2006. Nitrification kinetics of biofilm as affected by water quality factors. *Aquac Eng* 34: 179-197.

Clark E.R., Harman J.P. & Forster J.R.M. 1985. Production of metabolic and waste products by intensively farmed rainbow trout, Salmo gairdneri Richardson. *J Fish Biol* 27: 381-393.

Dalsgaard J., Larsen B.K. & Pedersen P.B. 2015. Nitrogen waste from rainbow trout (Oncorhynchus mykiss) with particular focus on urea. *Aquac Eng* 65: 2-9.

Dalsgaard J., Lund I., Thorarinsdottir R., Drengstig A., Arvonen K. & Pedersen P.B. 2013. Farming different species in RAS in Nordic countries: Current status and future perspectives. *Aquac Eng* 53: 2-13.

Davidson J., Good C., Welsh C. & Summerfelt S.T. 2014. Comparing the effects of high vs. low nitrate on the health, performance, and welfare of juvenile rainbow trout Oncorhynchus mykiss within water recirculating aquaculture systems. *Aquacult Eng* 59: 30-40.

DeLong D. & Losordo T. 2012. How to Start a Biofilter. *SRAC Publication No 4502* : 1-4.

Dworkin M., Falkow S., Rosenberg E., Schleifer K. & Stackebrandt E. 2006. *The Prokaryotes : A Handbook on the Biology of Bacteria.* Springer, Spring Street, New York, NY 10013 USA.

Farrell A. 2011. *Encyclopedia of fish physiology: from genome to environment.* Academic Press.

Fry B. 2006. *Stable Isotope Ecology.* Springer, Baton Rouge, LA 70803.

Green J.A., Hardy R.W. & Brannon E.L. 2002. The optimum dietary essential: nonessential amino acid ratio for rainbow trout (Oncorhynchus mykiss), which maximizes nitrogen retention and minimizes nitrogen excretion
 Kluwer Academic Publishers 27: 109-115.

Hagopian D.S. & Riley J.G. 1998. A closer look at the bacteriology of nitrification. *Aquac Eng* 18: 223-244.

Hart P.J. & Reynolds J.D. 2008. *Handbook of fish biology and fisheries.* John Wiley & Sons.

Holl C.M., Glazer C.T. & Moss S.M. 2011. Nitrogen stable isotopes in recirculating aquaculture for super-intensive shrimp production: Tracing the effects of water filtration on microbial nitrogen cycling. *Aquaculture* 311: 146-154.

Kankaala, P., Peura, S., Nykänen, H., Sonninen, E., Taipale, S., Tiirola, M., & Jones, R. I. (2010). Impacts of added dissolved organic carbon on boreal freshwater pelagic metabolism and food webs in mesocosm experiments.Fundamental and Applied Limnology/Archiv für Hydrobiologie, 177(3), 161-176.

Kuhn D.D., Drahos D.D., Marsh L. & Flick G.J. 2010. Evaluation of nitrifying bacteria product to improve nitrification efficacy in recirculating aquaculture systems. *Aquac Eng* 43: 78-82.

Likens G. 2009. *Encyclopedia of inland waters.* Academic Press.

Lucas J. & Southgate P. 2012. *Aquaculture: Farming aquatic animals and plants.* John Wiley & Sons.

Meier-Augenstein W. 2010. Stable Isotope Forensics: An Introduction to the Forensic Application of Stable Isotopes. *John Wiley & Sons, Inc* : 1-284.

Merbt S.N., Auguet J.-., Casamayor E.O. & Martí E. 2011. Biofilm recovery in a wastewater treatment plant-influenced stream and spatial segregation of ammonia-oxidizing microbial populations. *Limnol Oceanogr* 56: 1054-1064.

Michaud L., Lo Giudice A., Interdonato F., Triplet S., Ying L. & Blancheton J.P. 2014. C/N ratio-induced structural shift of bacterial communities inside lab-scale aquaculture biofilters. *Aquacult Eng* 58: 77-87.

Murray F., Bostock J. & Fletcher D. 2014. Review of Recirculating Aquaculture Systems Technologies and their Commercial Application. *Highlands and Islands Enterprise* : 1-82.

National Research Council. *Nutrient Requirements of Fish and Shrimp.* Washington, DC: The National Academies Press, 2011.

Ohashi A., De Silva D.G.V., Mobarry B., Manem J.A., Stahl D.A. & Rittmann B.E. 1995. Influence of substrate C/N ratio on the structure of multi-species biofilms consisting of nitrifiers and heterotrophs. *Water Science and Technology* 32 (8): pp. 75-84.

Pedersen L.-F., Oosterveld R. & Pedersen P.B. 2015. Nitrification performance and robustness of fixed and moving bed biofilters having identical carrier elements. *Aquac Eng* 65: 37-45.

Penczak T., Gallicka W., Molinski M., Kusto E. & Zalewski M. 1982. The enrichment of a mesotrophic lake by carbon, phosphorus and nitrogen from the cage aquaculture of rainbow trout, Salmo gairdneri (Glebokie Lake). *J Appl Ecol* 19: 371-393.

Pennell W. & Barton B. 1996. *Principles of salmonid culture.* Elsevier.

Schneider, K., Sher, Y., Erez, J., & van Rijn, J. (2011). Carbon cycling in a zero-discharge mariculture system. *Water Research, 45*(7), 2375-2382.

Silva, K., Wasielesky, W., & Abreu, P. (2013). Nitrogen and phosphorus dynamics in the biofloc production of the pacific white shrimp, Litopenaeus vannamei. *Journal of the World Aquaculture Society, 44*(1), 30-41.

Stenstrom M.K. & Poduska R.A. 1980. The effect of dissolved oxygen concentration on nitrification. *Water Res* 14: 643-649.

Stickney R. 1996. Aquaculture in the United States a Historical Survey. *John Wiley & Sons, Inc* : 371.

Summerfelt S.T., Zühlke A., Kolarevic J., Reiten B.K.M., Selset R., Gutierrez X. & Terjesen B.F. 2015. Effects of alkalinity on ammonia removal, carbon dioxide stripping, and system pH in semi-commercial scale water recirculating aquaculture systems operated with moving bed bioreactors. *Aquac Eng* 65: 46-54.

Timmons M. & Ebling J. 2010. *Recirculating Aquaculture.* Cayuga Aqua Ventures, 126 Sunset Drive, Ithaca, NY 14850.

Vielma, J., Mäkinen, T., Ekholm, P., & Koskela, J. (2000). Influence of dietary soy and phytase levels on performance and body composition of large rainbow trout (oncorhynchus mykiss) and algal availability of phosphorus load. *Aquaculture, 183*(3-4), 349-362.

von Ahnen M., Pedersen L.-F., Pedersen P.B. & Dalsgaard J. 2015. Degradation of urea, ammonia and nitrite in moving bed biofilters operated at different feed loadings. *Aquac Eng* 69: 50-59.

Wilderer P. 2011. *Treatise on Water Science.* IWA Publishing, Technische Universitaet Muenchen, Institute for Advanced Study, Munich, Germany.

Wuertz S., Schulze S.G.E., Eberhardt U., Schulz C. & Schroeder J.P. 2013. Acute and chronic nitrite toxicity in juvenile pike-perch (Sander lucioperca) and its compensation by chloride. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 157: 352-360.

Yokoyama, H., Abo, K., & Ishihi, Y. (2006). Quantifying aquaculture-derived organic matter in the sediment in and around a coastal fish farm using stable carbon and nitrogen isotope ratios. *Aquaculture, 254*(1-4), 411-425.

Zhu S. & Chen S. 1999. An experimental study on nitrification biofilm performances using a series reactor system. *Aquac Eng* 20: 245-259.